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Competence of *Agrobacterium rhizogenes* to accumulate heavy metals and toxicity of cadmium and copper to *Agrobacterium* strains

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ABSTRACT

Heavy metals cannot be degraded through various treatments. Bioaccumulation and bioaugmentation of heavy metals by food chain could damage normal physiological activity of higher organisms and threat human life. Heavy metals are very toxic even at low concentration (1.0 – 10 µg/ l) and their toxicity can last for a long time in nature. Short-deep treatment beds constructed at Abo-Attwa, Experimental Station, Ismailia, Egypt were efficient in the removal of organic matter, BOD and COD from primary treated influent by 50%, 66 % and 68 %, respectively. Also, the treatment beds were efficient in the removal of 93 – 98 % of the heavy metals Cu, Pb, Zn, Ni and Cd from influent. Eight *Agrobacterium* strains, three *A. rhizogenes* and five *A. tumefaciens*, were isolated and characterized from naturally-occurring microbial flora proximate domestic wastewater of Abo-Attwa Experimental Station, Ismailia, Egypt. The *Agrobacterium* strains were able to accumulate lead, zinc and nickel at varying rates. On the other hand, copper and cadmium were toxic to all *Agrobacterium* strains tested. The toxicity order of various heavy metals to *Agrobacterium* strains tested was Cd> Cu> Zn> Ni> Pb. The three *A. rhizogenes* strains accumulated higher amounts of Zn and Ni than *A. tumefaciens* strains. Induction of some tolerant *Agrobacterium* strains *via* growth adaptation in the presence of progressive high concentration of Cd and Cu was achieved. *A. rhizogenes* strain A_8 was superior in adaptation and survival as tolerant strain to both cadmium (Cd) and copper (Cu) on growing in Nutrient Broth containing elevated concentrations 1, 3, 10 and 30 µg/ l of both metals. The *A. rhizogenes* strains A_1 and A_7 efficiently accumulated 90 % more Cd than non-adapted *Agrobacterium* cells when grown at 10µg/l. Furthermore, The *A. rhizogenes* strains A_1, A_7 and A_8 accumulating 90 % , 75 % and 80 % respectively more Cu than non-adapted *Agrobacterium* cells when grown at 10µg/l. The bioaccumulation efficiency % of the *A. rhizogenes* strains A_1 and A_7 was 40 % for accumulating 30µg/l of the toxic metal cadmium (Cd). The potential role of Agrobacterium in the bioremediation of heavy metals was also considered.

Key words: *Agrobacterium*; Bioaccumulation, Biological treatment beds, Heavy metals; Tolerant.

INTRODUCTION

Metal accumulation in soil either from natural sources or from human activities, such as smelting, mining, processing, agricultural or industrial actions are leading to prominent risks due to leaching into surface and underground water, uptake by plants and direct or indirect intake by human population (Kärenlampi *et al*., 2000). River Nile, the most important source of drinking water in Egypt, is not secure from high levels of heavy metals. Soils around River Nile are also having high levels of heavy metals especially from using pesticides and fertilizers for decades (Elewa *et al.*, 1990).

When present at increased levels of bioavailability, both essential (Cu, Zn, Mn, Fe, Ni or Mo) and non-essential metals (Cd, Pb, Hg, or Cr) are toxic (Kärenlampi *et al*., 2000). The heavy metal contaminants of major concern are Cd, Cu, Zn, As and Pb, which arise, in excess of the permissible levels, from a number of industrial, mining or agricultural activities. The toxicity of heavy metals is an ecological and environmental problem.

Generally, heavy metals at excessive concentrations may have adverse effects on soil microbial biomass and activity. Short to medium-term toxic effects of heavy metals on soil microbial properties have been studied in soils where sewage sludge or sewage containing compost was applied as an organic fertilizer (Baath *et al*., 1998; Dewedar *et al*., 200٦). Microbial biomass is often below the levels expected from the amended organic carbon in heavy metals contaminated soils, partially due to the negative effects of heavy metals on soil microorganisms.

This study aims at determining the bioaccumulation efficiency of eight *Agrobacterium* strains, identified and characterized from a domestic wastewater treatment system, for various heavy metals. Overcoming toxicity of cadmium and copper to all *Agrobacterium* strains tested was endeavored by developing tolerant strains adapted through growing on elevated concentration of both metals.

MATERIALS AND METHODS

Chemicals used

Analytical pure heavy metals were used for the study including copper as $CuSO_45H_20$, cadmium as $Cd(NO_3)_2.4H_20$, zinc as $ZnSO_47H_2O$, lead as $Pb(NO_3)_2$, nickel as $NiSO_4$, mercuric as $HgCl_2$, silver as AgCl, manganese as $MnCl_2.4H_2O$ and chromium as K_2CrO_4 were purchased from Sigma Chemical Company.

Constructed wetland system

A constructed wetland system for the treatment of primary treated domestic wastewater was constructed at Abu-Attwa station, Ismailia, Egypt. It is comprised of six treatment beds (Fig. 1). The treatment beds offer the possibility of exploring a variety of conditions useful in

the process of optimization, such as detention time, filling media, treatment plants, water depth, etc. Phytodepuration efficiency was evaluated by comparing physical, chemical and microbiological characters of influent and effluents. Short-deep treatment beds are 20 m length, 2.5 m width, with different depths, and filled with gravel and/or sand as filling materials. Two beds were planted with *Cyperus papyrus*, while the other treatment beds were planted with *Phragmites australis* (Figure 1).

Samples from inlet and outlets were collected for heavy metal analysis

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Figure (1): Distribution system feeding primary-treated inlet (domestic wastewater) into six parallel treatment beds constructed at Abu-Attwa planted with *Cyperus papyrus* and *Phragmites australis*.

Sediment and water samples

Sediment and water samples were collected from the constructed wetland systems at Abu-Attwa Experimental Station, Ismailia, Egypt. The samples representing inlet, outlets, rhizosphere and rhizoplane were collected for physicochemical, microbiological and heavy metals analyses and for the isolation of *Agrobacterium* isolates.

Heavy metals extraction

Heavy metal extraction methods usually vary with the variation in sample type. Digestion of water and sediment samples was performed according to APHA standard methods (1998). Heavy metals extracted and digested from various samples were determined with atomic absorption spectrometry in comparison with authentic heavy metals concentractions.

physicochemical characteristics of domestic waste-water

Physical characteristics of wastewater collected from constructed wetland system at Abu-Attwa Station including temperature, dissolved oxygen and pH were measured on site during sample collection. Various chemical characteristics were performed according to the standard methods for the examination of water and wastewater (APHA, 1998).

Isolation, identification and characterization of *Agrobacterium*

Sediment samples were diluted in sterile distilled water and plated on *Agrobacterium* selective medium (Clark, 1969) plates containing 1µg/l Pb, Ni, Zn, Cd or Cu. Colonies showing resistance to any of the metals were selected. Isolation has been done to cover the possible different colonies according to their macro- and micro-morphological characteristics. Bacterial colonies were purified on the basis of their colonial characteristics on Nutrient Agar Medium for further studies. Identification of bacteria is based on a combination of growth characteristics, microscopic examination and biochemical characterization. The shape and colors of the isolated colonies were examined. Gram and spore staining cells were examined under the microscope. Several biochemical tests were performed on the isolates such as oxidase, catalase, gelatin hydrolysis, motility, indole production and citrate utilization. The series of biochemical tests were performed in order to build a phenotypic profile of each isolate according to Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986) and Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

Media for *Agrobacterium*.

Selective medium (Lactose 0.0, $Na_2HPO_4$1 ٨.l, KNO_3 1.0 , $MgSO_4.7H_2O$ 0.1and Agar 15.0 g/l)

Supplement Composition per 100 ml ($MnSO_4.4H_2O$ 3.35 Ferric EDTA 0.025 g/l)

Maintenance medium (Glucose 10.0 , Yeast extract 10.0, $(NH_4)_2SO$, 1.0 KH_2PO_4 0.25, Agar 15.0 g/l) (Clark, 1969).

Virulence test of *Agrobacterium* isolates against bioassay plant tissues

Discs from the tap roots of carrot *Daucus carota* as well as seedlings of tobacco *Nicotiana tabacum* were used to test the virulence of *Agrobacterium* isolates using a method recommended by (Pitzschke and Hirt, 2010). Proliferation of tumors or rhizoid formation was

investigated on carrot discs or tobacco seedlings inoculated with *Agrobacterium* isolates and compared with the test strains *A. rhizogenes* NRRL B-36 and *A. tumefaciens* NRRL B-193 obtained from the United State Department of Agriculture, Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA.

Opine analysis

Opines are low molecular weight compounds found in plant crown gall tumors or hairy roots produced by *Agrobacterium*. Opine biosynthesis is catalyzed by specific enzymes encoded by genes contained in a small segment of DNA (Known as the T-DNA, for transfer DNA), which is part of the Ti plasmid, inserted by the *Agrobacterium* into the plant genome. Opines are used by the *Agrobacterium* as an important source of nitrogen and energy. Each strain of *Agrobacterium* induces and catabolizes a specific set of opines. Opine analysis was performed in order to know the type of opine present in transformed plant tissues inoculated with various *Agrobacterium* isolates compared with standard opines (Tanaka *et al*., 1990). *Agrobacterium* isolates were compared with test strains *A. rhizogenes* NRRL B-36 and *A. tumefaciens* NRRL B-193 for opine type.

Cell growth measurement of *Agrobacterium* isolates.

After exposure of *Agrobacterium* strains grown in broth medium to five different concentrations (1, 3, 10, 30, 100 µg/l) of six different heavy metals (Pb, Ni, Zn, Cd and Cu) incubated at 27 ºC on an orbital shaker for 24 hours. Cell growth of *Agrobacterium* strains were determined by measurement of optical density at 550 nm with Orbeco-Hellige Digital direct-reading turbidimeter.

Agrobacterium survival % = mean of cell growth of *Agrobacterium* at given concentration of heavy metals / mean of normal cell growth of *Agrobacterium* (Yoshida *et al*., 1998).

Induction of tolerant *Agrobacterium* strains

Induction of tolerant *Agrobacterium* strains *via* growth adaptation in the presence of progressive high concentration of some heavy metals was attempt. Different heavy metals concentrations were added (1, 3, 10, 30, 100 µg/l) of five different heavy metals (Pb, Ni, Zn, Cd or Cu) to broth media of *Agrobacterium*. Subsequent subcultures of *Agrobacterium* strains were occurred from minor concentration of metals to the next higher one and *Agrobacterium* strains were incubated at 27 ºC for 48 h (Mullen *et al*., 1989).

Determination of antibiotic resistance

Resistance to antibiotics was determined on Nutrient Agar plates (Higgins *et al.*, 2001). Inhibition zone was measured after 48 h incubation at 25 ± 2°C. Strains were considered susceptible when the inhibition zone was ≥ 12 mm in diameter. The antibiotic sensitivity assay was performed as triplicates and the experiment was repeated three times. The following antibiotics Amoxil, Ampicillin, Chloramphenicol, Coftcin, Erythromycin, Garanycin, Oxytetracyclin, Penicillin, Rimactan, Streptomycin, Tetracyclin, Totacef, Unasyn and Vancocin were tested at 30 µg/ disc.

Statistical analyses

Analysis of results was done by one way analysis of variance (ANOVA) following by Post Hoc ANOVA Duncan test (Lentner and Bishop, 1986). Parameters monitored in the study were represented by the mean of three replicates and the standard deviations were calculated.

Results

Different types of pollutants contaminate wastewaters. Easily degradable pollutants or forms of pollution with a harmfulness which can be tolerated or reduced by the environment are included: pH, dissolved oxygen, BOD, COD, Organic matter, Total nitrogen and Total phosphorus. Pollutants with toxic action, but considered not accumulating in the aquatic ecosystems: boron, nickel, copper, and zinc. Pollutants which are thought to have a toxic and cumulative action: cadmium, mercury and lead. The ranges of physical and chemical properties of wastewater were determined and the results were presented in Table (1) as the mean of a whole year of the study.

Physicochemical analysis of inlet and outlet wastewaters from constructed wetland

The short-deep treatment beds constructed at Abo-Attwa, Experimental Station, Ismailia, Egypt were efficient in the removal of BOD and COD by 66 % and 68 %, respectively (Table 1). Also, beds are efficient in the removal of about 50 % of organic matter. On the other hand, beds can remove 93 – 98 % of the heavy metals Cu, Pb, Zn, Ni and Cd (Table 1).

Isolation and identification of heavy metal resistant *Agrobacterium*

In the present study we identified and characterized heavy metal resistant *Agrobacterium* isolated from sediment of beds treated primary treated domestic wastewater in the Experimental Station of Abo-Attwa, Ismailia, Egypt. One hundred colonies were screened from initial level of heavy metal supplemented culture medium. Eight strains were selected based on high degree of heavy metals and antibiotic resistances. The eight strains were used for the present study, which were identified as presented in Table (2). The ability of *Agrobacterium* isolates to infect plants and produce tumors or hairy roots was tested (Table 4). Virulence test of *Agrobacterium* to carrot discs was performed on all *Agrobacterium* strains as well as opine analysis (Table 3). Antibiotic resistance tests were also performed for differentiation among various *Agrobacterium* isolates Table (4) compared with two test strains. The *Agrobacterium* isolates (A1, A7 and A8) produced hairy roots on carrot discs and to tobacco

seedlings, the rest of the isolates produced tumors on carrot discs and tobacco seedlings incubated for 3 weeks at 25 ± 2 °C on MS medium (Table 4).

Therefore, three strains A1, A7 and A8 were identified eventually as *Agrobacterium rhizogenes* and five strains A2 – A6

Table (1): Average physicochemical parameters of constructed wetland beds feeding with primary treated influent and the resulting effluent complemented with removal efficiency percentage.

Parameters	Primary treated influent (± SD)	Effluent (± SD)	Removal efficiency %
pH	7.20 ± 0.11*	7.8 ± 0.16	
Dissolved oxygen (mg/l)	0.28 ± 0.15	1.3 ± 0.41	
Biochemical Oxygen Demand (mg/l)	141.65 ± 3.85	48.28 ±2.69	65.9 %
Chemical Oxygen Demand (mg/l)	657.18 ± 6.28	210.84 ± 5.66	67.9 %
Organic matter (mg/l)	42.73 ± 2.17	21.9 ± 1.37	48.7 %
Total nitrogen (mg/l)	32.83 ± 1.46	28.56 ± 1.97	13.1 %
Total phosphorus (mg/l)	1.1 ± 0.06	1.05 ± 0.08	4.5 %
Calcium (mg/l)	48.9 ± 2.55	37.74 ± 3.02	22.82 %
Boron (mg/l)	0.24 ± 0.12	0.20 ± 0.04	16.67 %
Sodium (mg/l)	4.07 ± 0.02	3.64 ± 0.02	10.57 %
Potassium (mg/l)	2.27 ± 0.03	2.16 ± 0.04	4.85 %
Copper (Cu) µg/ l	27.3 ± 2.3	1.2 ± 0.3	95.6 %
Lead (Pb) µg/ l	35.2 ± 2.1	2.3 ± 0.5	93.47 %
Zinc (Zn) µg/ l	180.1 ± 12.1	12.3 ± 3.2	93.17 %
Nickel (Ni) µg/ l	54.3 ± 1.2	0.55 ± 0.1	98.99 %
Cadmium (Cd) µg/ l	16.5 ± 0.02	1.0 ± 0.02	93.94 %

* Values are means of three replicate samples ± SD

Table (2): Identification and characterization of heavy metal resistant *Agrobacterium* isolates from soil at Abu-Attwa constructed wetland systems, Ismailia, Egypt

	Isolates							
Test	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**
Morphology								
Gram reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Spore stain	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile
Flagella arrangement								
Polar or subpolar	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Lateral	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Biochemical tests								
Oxidase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Urease	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Indole production	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Arginine dehydrolase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Caprate assimilation	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
Gelatine hydrolysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Citrate assimilation	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

Esculin hydrolysis	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Gas from Carbohydrates	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
H_2S from cysteine	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
n-acetyl-glucosamine	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Phenyl-acetate	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
B-galactosidase	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve
Nitrate reduction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Nitrite reduction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Assimilation								
Glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Malate	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Maltose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Mannitol	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Mannose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Adipate	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve
Arabinose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Gluconate	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

Table (3): Infectivity of *Agrobacterium* isolates and/ or strains to *Daucus carota* discs and *Nicotiana tabacum* seedlings and opines analysis for different *Agrobacterium* isolates compared to test strains

***Agrobacterium* isolate/ Strain**	**Opine type**	**Virulence test against plants**	
		Daucus carota	*Nicotiana tabacum*
A. rhizogenes NRRL B-36 *	Agropine	Hairy roots	Hairy roots
A. tumefaciens NRRL B-193*	Octopine	Tumors	Tumors
A. rhizogenes A 1	Agropine	Hairy roots	Hairy roots
A. tumefaciens A 2	Octopine	Tumors	Tumors
A. tumefaciens A 3	Octopine	Tumors	Tumors
A. tumefaciens A 4	Octopine	Tumors	Tumors
A. tumefaciens A 5	Octopine	Tumors	Tumors
A. tumefaciens A 6	Octopine	Tumors	Tumors
A. rhizogenes A 7	Agropine	Hairy roots	Hairy roots
A. rhizogenes A 8	Agropine	Hairy roots	Hairy roots

* Test strains obtained from the United State Department of Agriculture, Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA.

Table (4): Antibiotic resistance test for various *Agrobacterium* strains against fourteen different antibiotics

Antibiotic Sensitivity test (30 µg/disc)	**Diameter of Inhibition zone (mm) against *Agrobacterium* strains**							
	***A. rhizogenes* A 1**	***A. tumefaciens* A 2**	***A. tumefaciens* A 3**	***A. tumefaciens* A 4**	***A. tumefaciens* A 5**	***A. tumefaciens* A 6**	***A. rhizogenes* A 7**	***A. rhizogenes* A 8**
Amoxil	14 (S) **	12 *	6 (R)	6 (R)	6 (R)	7 (R)	6 (R)	16 (S)
Ampicillin	-ve	-ve	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	9 (R)
Chloramphenicol	21 (S)	34 (S)	31 (S)	41 (S)	33 (S)	25 (S)	31 (S)	33 (S)
Coftcin	11 (R)	22 (S)	18 (S)	46 (S)	42 (S)	19 (S)	23 (S)	26 (S)
Erythromycin	6 (R)	25 (S)	7 (R)	19 (S)	35 (S)	6 (R)	13 (S)	29 (S)
Garanycin	7 (R)	13 (S)	9 (R)	9 (R)	16 (S)	11 (R)	11 (S)	24 (S)
Oxytetracyclin	39 (S)	38 (S)	21 (S)	27 (S)	45(S)	26 (S)	34 (S)	39 (S)
Pencillin	-ve	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	-ve
Rimactan	20 (S)	36 (S)	24 (S)	36 (S)	32 (S)	22 (S)	34 (S)	26(S)
Streptomycin	12 (R)	22 (S)	8 (R)	23 (S)	31 (S)	22 (S)	28 (S)	26 (S)
Tetracyclin	-ve	37 (S)	-ve	36 (S)	35(S)	7 (R)	11 (R)	23 (S)
Totacef	-ve	13 (S)	26 (S)	6 (R)	15 (S)	8 (R)	18 (S)	12 (R)

Unasyn	-ve	12 (R)	-ve	13 (S)	17 (S)	-ve	-ve	6 (R)
Vancocin	20 (S)	34 (S)	24 (S)	25 (S)	27 (S)	24 (S)	27 (S)	29 (S)

* *Agrobacterium* strain was considered resistant when the inhibition zone against an antibiotic was ≤ 12 mm

** *Agrobacterium* strain was considered sensitive when the inhibition zone against an antibiotic was > 12 mm

at 25 ± 2 °C on MS medium (Table 4). Therefore, three strains A1, A7 and A8 were identified eventually as *Agrobacterium rhizogenes* and five strains A2 – A6 as *Agrobacterium tumefaciens* on performing all the above mentioned identification and characterization tests and comparing with the test strains *A. rhizogenes* NRRL B-36 and *A. tumefaciens* NRRL B-193 (Table 4).

Heavy metal concentration in inlet and outlets from wastewater treatment system.

Various heavy metals (Cu, Pb, Zn, Ni, Cd and Ag) were extracted from inlet and outlets of wastewater of the biological treatment system and the results are presented in Table (1). Levels of heavy metals present in primary treated inlet were 180.1 µg/l Zn, 54.3 µg/l Ni, 35.2 µg/l Pb, 27.3 µg/l Cu and 16.5 µg/l Cd. Amounts of the heavy metals accumulated in the inlet of biological treatment system were in the order of Zn >Ni >Pb >Cu >Cd. The biological treatment beds were efficient in the removal of 95.6, 93.47, 93.17, 98.99 and 93.94 % of the heavy metals Cu, Pb, Zn, Ni and Cd as follows, respectively (Table 1).

The levels of heavy metals in soil samples that used for isolation of *Agrobacterium* strains.

The levels of the heavy metals Zn, Cu, Cd, Ni, Pb and Ag accumulated in soil samples (Fig. 2) showed that concentrations of Cu, (26.85 µg/g) Pb, (114.25 µg/g) Zn, (50.7 µg/g) Ni, (47.5 µg/g) and Cd (2.4 µg/g). Concentration of the heavy metals in the soil samples were in the order of Pb > Zn > Ni > Cu. Very low amount of Cd was found in such soil samples, while negligible amount of Ag was determined in soil samples.

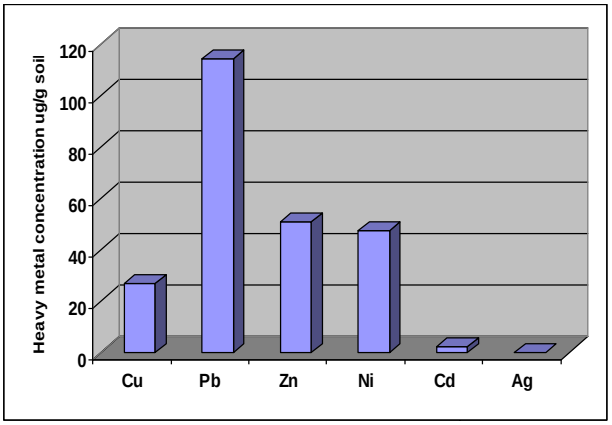


Figure (2): Concentration of heavy metals (µg/g) in sediment samples that used for the isolation of *Agrobacterium*strains.

Concentration of heavy metals in *Agrobacterium* cells.

The levels of the heavy metals (Pb, Ni, Zn, Cd or Cu) accumulated in the cells of eight *Agrobacterium* strains (A1, A2, A3, A4, A5, A6, A7 and A8) were determined and the results were presented in Fig. (3). All *Agrobacterium* strains accumulated lead (Pb) at more or less the same levels. *Agrobacterium rhizogenes* strains A_1, A_7 and A_8 accumulating higher amounts of Ni compared to the *Agrobacterium tumefaciens* strains A_2, A_3, A_4, A_5 and A_6. Moreover, *Agrobacterium rhizogenes* strains A_1, and A_8 accumulating high amounts of Zn. Therefore, the *Agrobacterium rhizogenes* strains were able to accumulate the heavy metals Ni and Zn more efficiently than *Agrobacterium tumefaciens* strains. On the other hand, it was quite clear that all *Agrobacterium* strains were not able to accumulate the metals Cu and Cd.

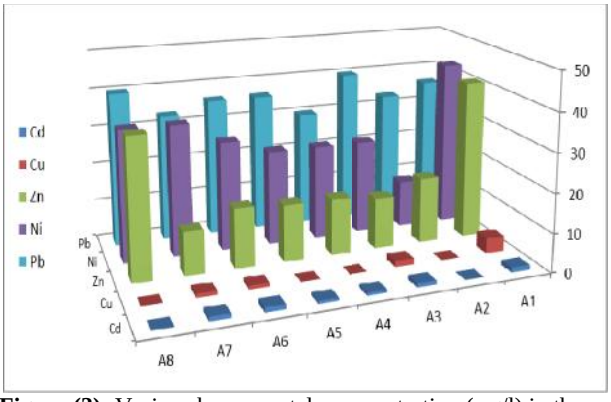


Figure (3): Various heavy metals concentration (µg/l) in the cells of *Agrobacterium* strains isolated from a biological wastewater treatment system.

Results in the pie charts (Fig. 4) showed that percentage accumulations of the five heavy metals Pb, Ni, Zn, Cd and Cu in the cells of various *Agrobacterium* strains were varying in the levels of accumulation corresponding to the levels of accretion of other metals.

Induction of tolerant *Agrobacterium* strains to cadmium and copper

Growth was measured photometrically at optical density 550 nm. The measurements were taken by a digital direct-reading turbidimeter after adding separately five different concentrations (1, 3, 10, 30, 100 µg/l) of six different heavy metals cadmium (Cd) and copper (Cu) to broth media of *Agrobacterium* incubated at 27 ºC (Figure 5).

The measurements of metals accumulation were measured by atomic absorption spectrometry after adding five different concentrations (1, 3, 10, 30, 100 µg/l) of Cd and Cu to broth media of *Agrobacterium*. The adapted cells were developed by subsequent subcultures of *Agrobacterium* strains from minor concentration of metals (1 µg/l) to the next higher one and incubation of *Agrobacterium* strains at 27 ºC, while the various heavy metals concentration (µg/l) in the

cells of *Agrobacterium* strains strained from a biological wastewater treatment system concentrations represented the non-adapted cells (control for uptake of heavy metals without adaptation). It was quite clear that the non-adapted cells (control) accumulated the metals at lower levels of Cd and Cu uptake efficiency, while the uptake efficiency of adapted cells of all *Agrobacterium* strains at 10, 30 and 100 µg/l accumulated the metals at higher levels corresponding to the levels of control (Figure 5). The levels of uptake efficiency at 10µg/l were the highest one due to the effect of toxicity of metals on *Agrobacterium* strains at 30 and 100µg/l. *Agrobacterium rhizogenes* strain A_8 is superior in adaptation and survival as tolerant strain to both cadmium (Cd) and copper (Cu) on growing in elevated concentrations 1, 3 and 10 µg/ l of Cd or Cu metals. *Agrobacterium rhizogenes* strain A_8 survived ten times better than other tested strains (Figure 5).

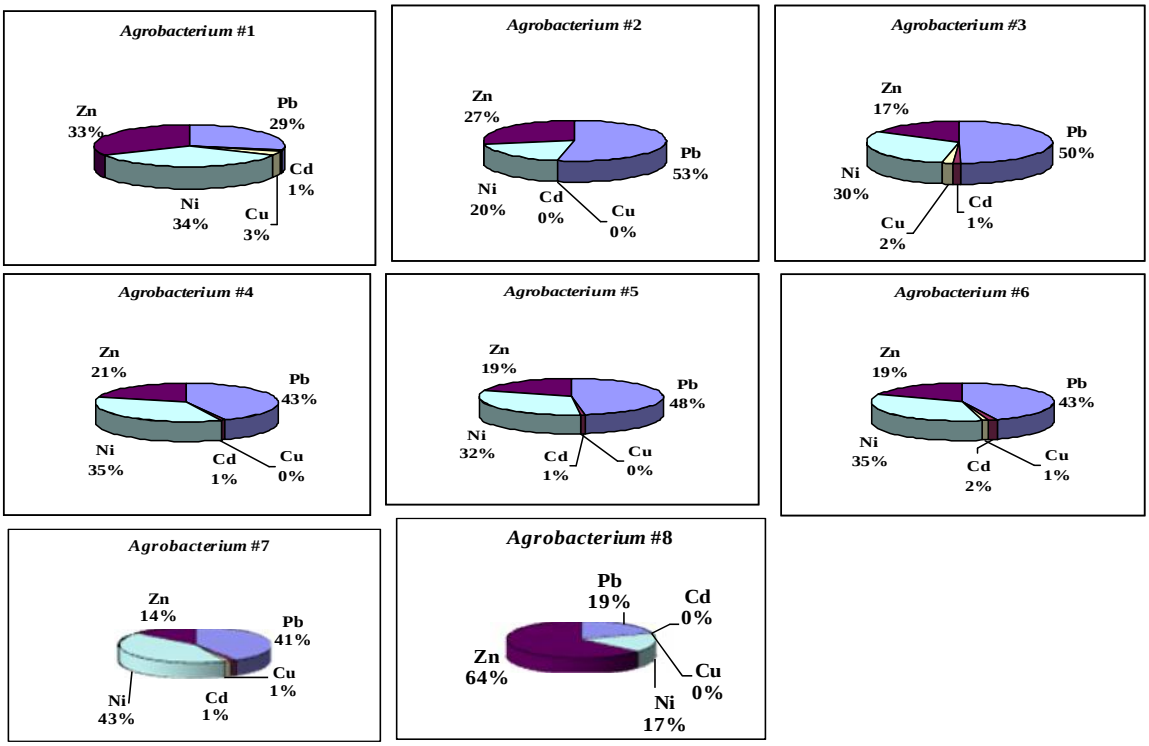
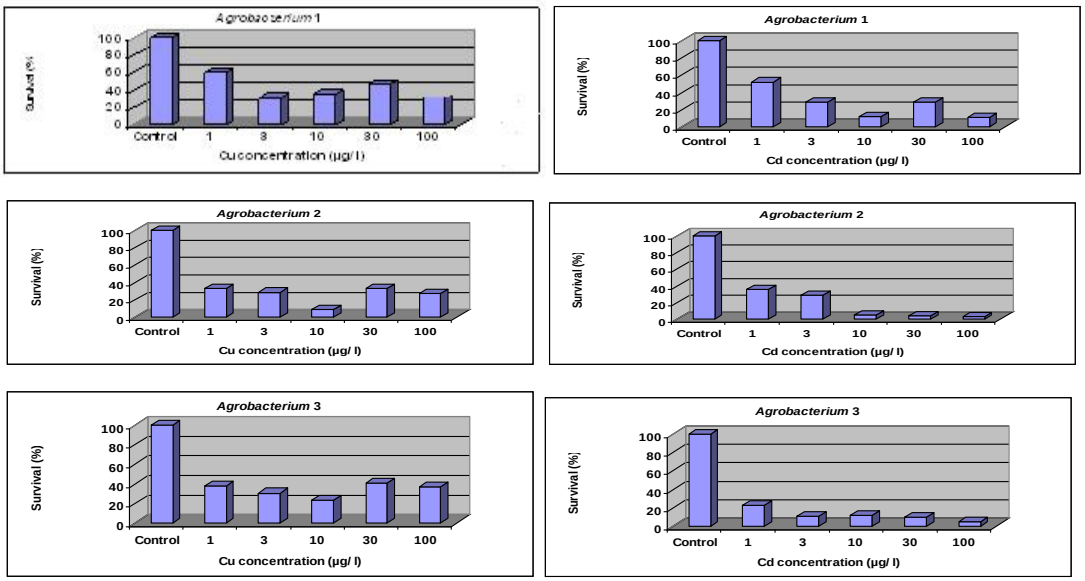


Figure (4): Various heavy metals concentration percentage for each strain in the cells of *Agrobacterium* isolated from a biological wastewater treatment system.



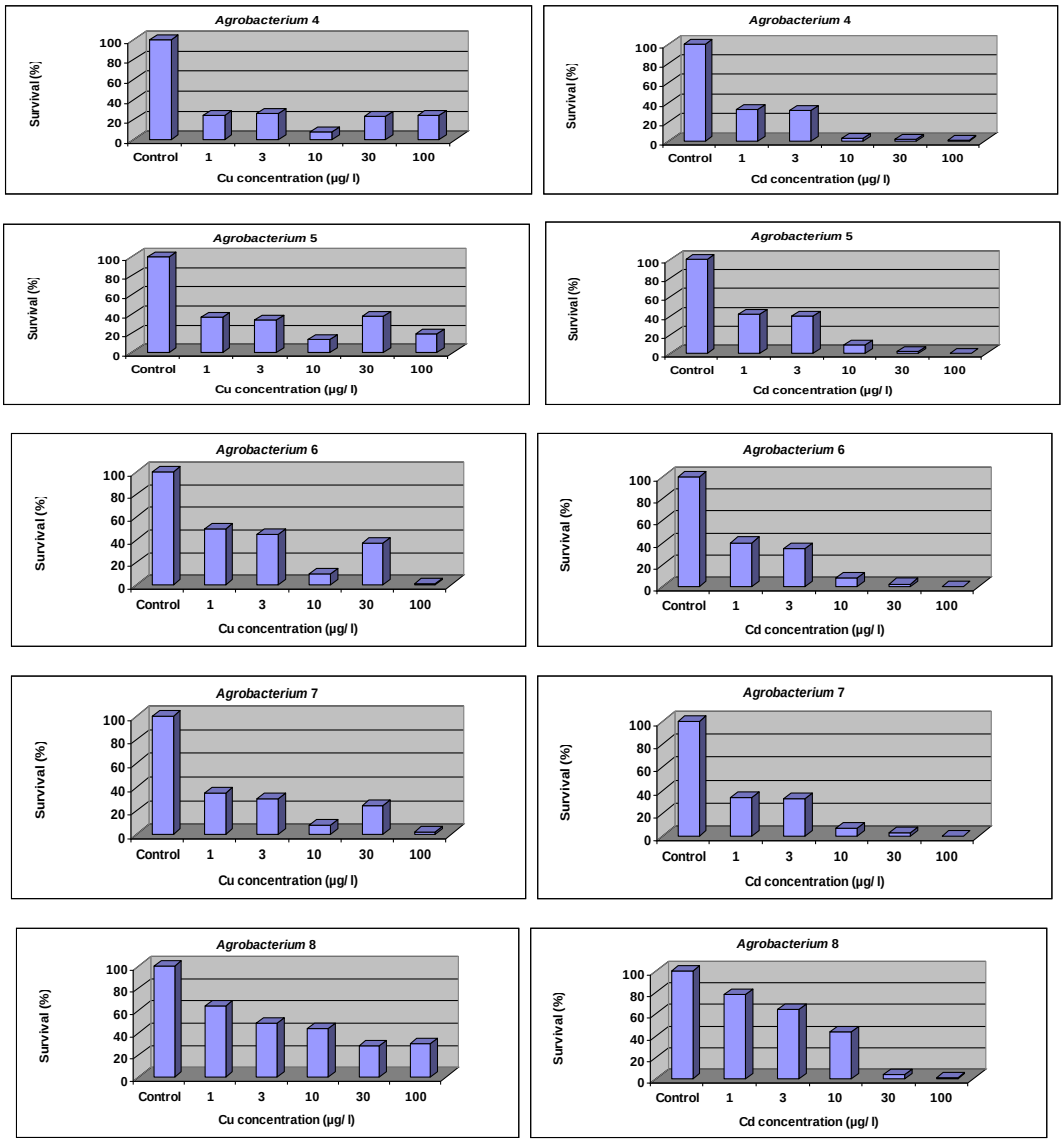


Figure (5): Cell growth of *Agrobacterium* strains in response to various concentrations of heavy metals (Cu and Cd) in broth medium.

Bioaccumulation efficiency % of Cadmium (Cd) and/ or copper (Cu) for *Agrobacterium* strains

Results in Figure (6) presents the bioaccumulation efficiency % of the *Agrobacterium rhizogenes* strains A_1 and A_7 for accumulating 90 % more Cd than non-adapted *Agrobacterium* cells when grown at 10µg/l. Bioaccumulation efficiency % of *Agrobacterium rhizogenes* strain A_8 and *Agrobacterium tumefaciens* strain A_6 for Cd was 76 % when grown at 10µg/l. Bioaccumulation efficiency % of the *Agrobacterium rhizogenes* strains A_1, A_7 and A_8 for accumulating 90 % , 75 % and 80 % respectively more Cu than non-adapted *Agrobacterium* cells when grown at 10µg/l. Bioaccumulation efficiency % of *Agrobacterium tumefaciens* strains A_3 and A6 for Cu was 82 % and 75 %, respectively when grown at 10µg/l.

On the other hand, bioaccumulation efficiency % of the *Agrobacterium rhizogenes* strains A_1 and A_7 was 40 % for accumulating 30 µg/l of the toxic metal cadmium

DISCUSSION

It is well recognized that microorganisms have a high affinity for metals and can accumulate heavy metals by a variety of mechanisms. Bacteria, fungi, and action-mycetes are highly effective in sequestering heavy metals and consequently can be used to remove metals from polluted industrial and domestic effluent on a large scale (Miller and Poindexter, 1994).

The bioremediation of heavy metals by micro-organisms is an alternative tool to chemical methods. The microbial remediation is usually the only means for complete mineralization of organic molecules in water and soils (Gibson, 1984).

In the present study, eight *Agrobacterium* strains were identified. Bacterial identification was carried out using standard phenotypic characterization and was supported by a bioassay for phytopathogenicity. Three strained bacterium were *A. rhizogenes* and four strains were *A. tumefaciens* (Rashad, 2005).

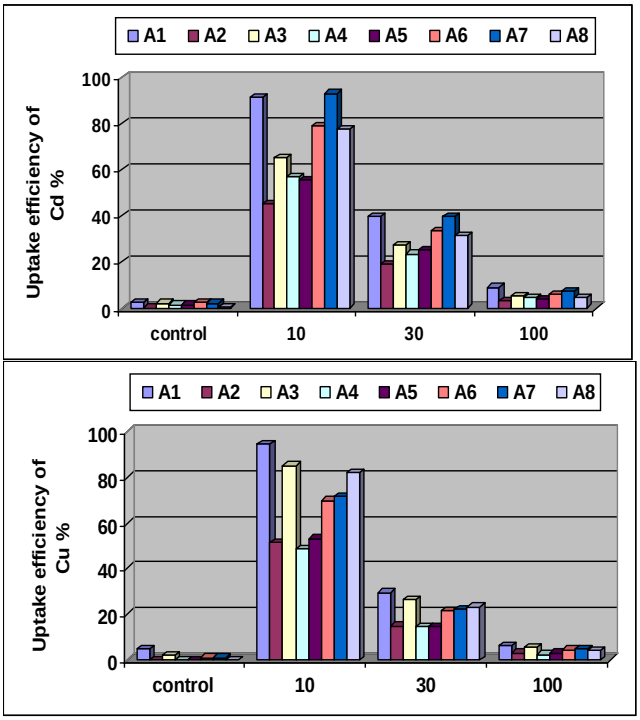


Figure (6): Bioaccumulation efficiency % of various heavy metals in *Agrobacterium* cells after growing on successive concentrations.

Agrobacterium rhizogenes strains accumulated higher amounts of Zn and Ni than *A. tumefaciens* strains. The *Agrobacterium rhizogenes* strain A_8 was superior in adaptation and survival as tolerant strain to both cadmium (Cd) and copper (Cu) on growing in elevated concentrations 1, 3 and 10 µg/ l of Cd or Cu metals.

The present study find that the toxicity of the heavy metals to *Agrobacterium* strains was in the following order Cd > Cu > Zn > Ni > Pb and this was in agreement with Baath (1992) who ranked the toxicity of five metals on soil microbes as Ag > Cu > Cd > Zn > Pb. In a field experiment, Aoyama and Nagumo (1997) gave toxic order as Cu > Pb > As. Metal-tolerant bacteria are not as fast growing as at least some of the metal-sensitive ones. Moreover, metal-tolerant bacteria have an energetic burden which will be a competitive disadvantage during conditions of normal soil metal concentrations. The rapid response of the non-tolerant microorganisms during recolonization indicated that they were already present, although in low numbers, in polluted soils.

A practical method of producing more metal resistant and efficient strains is through adaptation of the cells to progressively higher concentrations of heavy metals. Adapted cells could grow well in presence of higher metal concentration while the non-adapted cells perished. Moreover, the specific metal uptake capacity and the metal removal by adapted cells were higher than the non-adapted cells at all the concentrations tested. Bacteria have been produced tolerant strains *via* adaptation (Malik, 2004).

The comparison of effects observed when metals were added directly to the soil *versus* when metals were added to suspensions of bacteria extracted from soil illustrates two points. The first is well known: physical and chemical interactions of metals with soil reduce the bioavailability of metals. Therefore, the mass of metal required to inhibit microbial activity was several orders of magnitude higher in soil than in suspensions. These interactions were one of the reasons for using phytoremediation. However, the unexpected similarities of tolerance levels found in bacteria extracted from soils with high and low metal contents prompted a direct examination of effects in soil (Shi *et al*., 2002).

Brierley (1990) have described the many ways in which bacteria, can take up toxic metal ions. Heavy-metal ions can be entrapped in the cellular structure and subsequently biosorbed onto the binding sites present in the cellular structure. This method of uptake is known as ‘‘biosorption’’ or ‘‘passive uptake’’. The heavy metal can also pass into the cell across the cell membrane through the cell metabolic cycle. This mode of metal uptake is referred to as ‘‘active uptake’’. The metal uptake by both active and passive modes can be termed as ‘‘bioaccumulation’’.

Russell *et al*., (1998) strained *Methylobacterium*, *Variovorax*, *Enterobacter*, *Aureobacterium*, and *Bacillus* from heavy metal polluted soil. The mixed culture biodegraded metal-EDTA complexes slowly. The biodegradability was in the order Fe > Cu > Co > Ni > Cd. Significant removal of complexes by an *Agrobacterium* species was also demonstrated (Russell *et al*., (1998).

In conclusions a practical method of producing more metal resistant and efficient strain is through adaptation of the cells to progressively higher concentrations of heavy metals. The specific metal uptake capacity and the metal removal by adapted *Agrobacterium* cells were higher than the non-adapted cells at all the concentrations tested. Bacteria have been produced tolerant strains *via* adaptation. *Agrobacterium* strains tested were not able to accumulate the metals Cu and Cd. The uptake efficiency of adapted *Agrobacterium* strains at 10 µg/g accumulated the metals at higher levels than the control.

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مقدرة الأجروبكتريمريزوجينز على تراكم المعادن الثقيلة وسمية الكادميوم والنحاس لعزلات الأجروبكتريم

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الملخـــص العربـــي

تستعصى المعادن الثقيلة على المعالجات المختلفة التى تستهدف تكسيرها. كما تدمر المعادن الثقيلة العمليات الفسيولوجية المختلفة لجميع الكائنات الحية عند دخولها السلسلة الغذائية فى الطبيعة، مما يهدد حياة الانسان. تسبب المعادن الثقيلة سمية للكائنات الحية حتى عند التركيزات الضئيلة ١ – ١٠ ميكرو جرام/ لتر، كما تظل سميتها لوقت طويل فى الطبيعة.

تم عزل وتعريف ثمانية عزلات من الأجروبكتريم، لها القدرة على تراكم المعادن الثقيلة بخلاياها، من المحطة التجريبية لمعالجة مياه الصرف الصحي بأبي عطوه – الاسماعيلية – مصر. وقد وجد أن عزلاتالأجروبكتريم الثمانية تتفاوت فى قدرتها على تراكم الرصاص والزنك والنيكل، ولكن جميع العزلات لا تستطيع أخذ الكادميوم أو النحاس. تم عمل تجربة لرفع كفاءة العزلات الثمانية بنموها فى وسط غذائي سائل يحتوى على تركيزات متصاعدة من كل معدن من المعادن الثقيلة المراد رفع كفاءتها حتى تستطيع تراكمه فى خلاياها بنسبة أكبر.

وقد ثبت أن عزلات الأجروبكتريمريزوجيز متفوقة على عزلات الأجروبكتريمتوميفاسنز فى تراكم الزنك والنيكل، كما أن لها القدرة الأعلى على التكيف وتراكم الكادميوم والنحاس بعد تنميتها على تدرجات متنامية من الكادميوم أو النحاس.

تم مناقشة مقدرة الأجروبكتريم على المعالجة الحيوية للمعادن الثقيلة وكذلك تمت الاشارة للوعى بسمية المعادن الثقيلة على الكائنات الدقيقة والكائنات الراقية لتلافى أخطار تلك السمية على الانسان والبيئة.